

Irradiation-Induced Telomerase Activity and the Risk of Lung Cancer

A Pilot Case-Control Study

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BACKGROUND. Telomerase activity is undetectable in most normal somatic cells, but is up-regulated by various mechanisms during tumorigenesis. Telomerase activation enables cells to overcome replicative senescence and maintain telomere stability during cell proliferation. The aim of the study was to evaluate the association between irradiation-induced telomerase activity and the risk of lung cancer.

METHODS. A case-control design was used that measured the baseline and γ -radiation-induced telomerase activity in cultured peripheral blood lymphocytes from 44 lung cancer patients and 44 healthy controls. The associations between γ -radiation-inducible telomerase activity and the risk of lung cancer were then analyzed.

RESULTS. The baseline telomerase activity was lower in cases than in controls (0.956 vs 1.222, $P = .126$). After γ -radiation the telomerase activity in cases experienced a significant increase over baseline (1.480 vs 0.956, $P < .001$); the telomerase activity in controls also increased, but on a smaller scale (1.485 vs 1.222, $P = .0025$). The relative γ -radiation-induced telomerase activity, defined as the ratio of the net increase of telomerase activity (γ -radiation induced minus baseline) to the baseline telomerase activity, was significantly higher in cases than in controls (0.730 vs 0.224, $P = .0003$). When dichotomized, the subjects at the 75th percentile of the relative γ -radiation-induced telomerase activity in controls, a higher ratio was associated with a significantly increased lung cancer risk (odds ratio [OR], 4.71, 95% confidence interval [CI]: 1.37, 16.21). Moreover, a dose response was observed between the relative γ -radiation-induced telomerase activity and lung cancer risk. Compared with individuals with the lowest tertile of the relative γ -radiation-induced telomerase activity, individuals with the second and the highest tertiles of the relative telomerase activity exhibited significantly elevated risks of lung cancer, with adjusted ORs of 12.58 (95% CI: 1.08, 146.86) and 31.08 (95% CI: 2.71, 356.81), respectively (P for trend $< .001$).

CONCLUSIONS. The pilot-case control study suggested that the γ -radiation-induced telomerase activation is associated with a significantly increased risk of developing lung cancer. Larger case-control studies and prospective studies are needed to confirm the findings. *Cancer* 2007;109:1157-63.

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KEYWORDS: telomerase activity, lung cancer, susceptibility.

Telomerase, a ribonucleoprotein complex, is responsible for telomere elongation and maintenance, as well as chromosome stability during cell proliferation.^{1,2} Telomerase is activated in over 85% of cancer cells, but undetectable in the majority of normal somatic cells.^{3,4} Moreover, inhibition of telomerase activity limits cancer cell

growth and leads to apoptosis,⁵⁻⁷ which indicates that telomerase plays a crucial role in cellular immortalization and tumorigenesis.

The up-regulation of telomerase activity has been shown to be triggered by p53-related DNA damage response by both molecular biological and epidemiological studies.⁸⁻¹⁰ Radiation induces DNA breaks, triggers the p53 damage response pathway, and stimulates telomerase activity¹¹⁻¹⁴ in normal cells. More directly, a dose-dependent increase in telomerase activity was observed after irradiation in human lymphoblasts.¹⁴

Given the prevalence of telomerase in tumors and its absence in normal somatic cells, telomerase has been widely studied as a biomarker for the diagnosis and prognosis of cancer and as a target for cancer therapy.¹⁵⁻¹⁷ However, there have not been any reports evaluating telomerase activity as a potential susceptibility factor for cancer. We hypothesized that cancer cases are more susceptible to γ -radiation-induced telomerase activation than healthy controls and that γ -radiation-induced telomerase activity may be a predisposing factor for lung cancer. We tested the hypothesis in our ongoing lung cancer case-control study by comparing the baseline and γ -radiation-induced telomerase activity in cultured peripheral blood lymphocytes (PBLs) from lung cancer patients and matched healthy controls.

MATERIALS AND METHODS

Study Population

The cases were recruited from the University of Texas M. D. Anderson Cancer Center through a daily review of computerized appointment schedules. The cases were chosen consecutively from our ongoing lung cancer case-control study. Among the cases, 9 had early stage lung cancer (stages 1 and 2), 31 had late stage lung cancer (stages 3 and 4), and 4 had unspecified stage. The cases were diagnosed within 1 year of recruitment with histologic confirmation of lung cancer. Patients with a medical history of prior chemotherapy or radiotherapy were excluded. The control subjects were healthy individuals with no prior history of cancer (except nonmelanoma skin cancer) who were recruited from Kelsey Seybold, the largest multispecialty physician group in the Houston metropolitan area. Control subjects were matched to the case patients by age (± 5 years), gender, and ethnicity. In this study, all subjects were Caucasians. The information about demographics, smoking, alcohol consumption, and medical history were collected from all participants. Signed informed consents were obtained from all subjects. This study was approved

by the Institutional Review Boards of M. D. Anderson Cancer Center and Kelsey Seybold Clinic.

Blood Collection, Lymphocyte Isolation, and Cell Culture

Immediately after the interview, 40 mL of blood was collected into heparinized tubes. Lymphocytes were isolated by Ficoll-Hypaque centrifugation and aliquots of 4×10^6 isolated lymphocytes per vial were stored in liquid nitrogen as previously described.¹⁸ The frozen lymphocytes were thawed and cultured in RPMI 1640 media supplemented with 20% heat-inactivated fetal bovine serum and 112.5 $\mu\text{g/mL}$ phytohemagglutinin. Cells were incubated at 37°C for 72 hours in a humidified atmosphere containing 5% CO₂. All experiments were carried out using exponential phase growing cells. Cultured lymphocytes were irradiated with γ -radiation at a predetermined optimal dose of 0.5 Gy from a ¹³⁷Cs source (Cesium Irradiator Mark 1, model 30; J. L. Shepherd and Associates, Glendale, CA). Cells were allowed to grow for an additional 12 hours after exposure. Cells were then harvested, resuspended in 200 mL of lysis buffer, and incubated for 30 minutes on ice. Unirradiated cells were also harvested at the same time point. After centrifugation at 16,000g for 20 minutes at 4°C the supernatants were collected. The protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA).

Telomerase Activity Measurement

Telomerase activity was assessed using the telomerase polymerase chain reaction (PCR) enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Briefly, the supernatants containing equal amount of proteins were incubated with biotinylated telomerase substrate oligonucleotide (P1-TS primer) at 25°C for 20 minutes. The extended products were amplified by PCR using P2 primers for 30 cycles under the following conditions: 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds. Biotinylated TRAP products were incubated for 2 hours at 37°C with a digoxigenin-labeled detection probe complementary to the telomeric repeat sequence and immobilized onto streptavidin-coated microtiter plates. The wells were further incubated at room temperature for 30 minutes with peroxidase-labeled anti-digoxigenin polyclonal antibody. Finally, the amount of TRAP products was determined after the addition of the peroxidase substrate (3,3',5,5'-tetramethylbenzidine). The absorbance of each sample was measured at a wavelength of 450 nm (reference wavelength, 595 nm; Vmax Kinetic Microplate Reader, Molecular Devices, Sunnyvale, CA). For each

sample a heat-treated negative control was included. Relative telomerase activity in each sample was defined in the following way: (absorbance of sample – absorbance of heat-treated sample)/absorbance of internal standard sample. The laboratory personnel were blinded to the case-control status of the subject.

Statistical Analysis

Telomerase activity was analyzed both as a continuous variable and as a categorical variable. The χ^2 test was used to test for differences in distribution between the cases and controls with regard to gender and smoking status. The Wilcoxon rank sum test was conducted to compare the differences between cases and controls for continuous variables, including age, pack-years, and telomerase activity. The relative telomerase activity was also analyzed as a categorical variable by dichotomizing the subjects at the 75th percentile of the relative telomerase activity in controls, or by grouping the subjects according to the tertile distribution of the relative telomerase activity in controls. To control the confounding, unconditional multiple logistic regression analysis was conducted and adjusted odds ratios (ORs) along with 95% confidence intervals (CIs) were calculated with STATA software (College Station, TX). All statistical tests were 2-sided.

RESULTS

A total of 44 cases and 44 controls were included in this study. Table 1 shows selected characteristics of the cases and controls. They were well matched on age and gender. The cases had a significantly higher percentage of former (50.00%) and current (47.73%) smokers than the controls (38.64% and 9.09%, respectively) ($P < .001$). Cases had a heavier smoking history than controls (mean pack-years: 58.85 vs 12.37, $P < .001$).

We measured the baseline and γ -radiation-induced telomerase activities in PBLs using the telomerase PCR-ELISA assay (Table 2). The baseline telomerase activity was lower in cases than in controls (0.956 vs 1.222, $P = .126$), although the difference was not statistically significant. To select an optimal dosage of γ -radiation, we treated a control lymphocyte sample with various dosage of γ -radiation. As shown in Figure 1, the relative telomerase activity for nontreated, 0.25, 0.5, 1, and 2 Gy γ -radiation-treated PBLs was 1.03 ± 0.12 , 1.12 ± 0.06 , 1.31 ± 0.21 , 1.96 ± 0.25 , and 1.81 ± 0.33 , respectively. There was a clear dose-response effect when the γ -radiation dose was between 0 and 1 Gy. The induction of telo-

TABLE 1
Distribution of Select Characteristics of Cases and Controls

Variables	Cases no. (%)	Controls no. (%)	<i>P</i> *
Sex			
Men	31 (70.45)	31 (70.45)	
Women	13 (29.55)	13 (29.55)	1.000
Age, y			
Mean [SD]	65.02 [8.92]	65.23 [8.76]	.914
Smoking status			
Never	1 (2.27)	23 (50.27)	
Former	22 (50.00)	17 (38.64)	
Current	21 (47.73)	4 (9.09)	<.001
Pack-years			
Mean [SD]	58.85 [38.71]	12.37 [19.60]	<.001

* *P* derived from the Pearson χ^2 test for categorical variables (sex and smoking status) and the Wilcoxon rank sum test for continuous variables (age and cigarette smoking pack-years). All *P* 2-sided.

merase reached a plateau when the γ -radiation dose was >1 Gy. We selected 0.5 Gy as the dosage for the following case-control study because it was at the midpoint of the linear portion of the dose-response plot (Fig. 1). After γ -radiation, the telomerase activity in cases experienced a significant increase over baseline (1.480 vs 0.956, $P < .001$), whereas the telomerase activity in controls increased on a smaller scale (1.485 vs 1.222, $P = .0025$). The relative γ -radiation-inducible telomerase activity, defined as the ratio of the net increase of telomerase activity (γ -radiation induced minus baseline) to the baseline telomerase activity, was significantly higher in cases than in controls (0.730 vs 0.224, $P = .0003$).

We then performed unconditional logistic regression analysis to assess the association between the relative γ -radiation-inducible telomerase activity and the risk of lung cancer. We used the 75th percentile value of the relative γ -radiation-inducible telomerase activity in controls as the cutoff point to dichotomize the subjects into low and high ratio groups (Table 3). After adjusting for age, gender, and smoking status, individuals with a higher ratio were associated with a significantly increased lung cancer risk (OR, 4.71, 95% CI: 1.37, 16.21). Because smoking details might affect lymphocyte proliferation and telomerase phenotype, we also performed this analysis by adjusting for pack-years. The resulting OR was 12.88 (95% CI, 12.56, 64.78). The larger risk estimate and wider CI were mostly likely due to small sample size and wide range of pack-years.

We further categorized subjects into tertiles of the ratio of telomerase activity based on its distribution in the controls. A dose response was observed

TABLE 2
Telomerase Activity in Cases and Controls

Telomerase activity	No.	Cases		No.	Controls		<i>P</i> [†]
		Mean (SD)	<i>P</i> [*]		Mean (SD)	<i>P</i> [*]	
Baseline	44	0.956 (0.704)	—	44	1.222 (0.901)	—	.1260
After γ -radiation treatment	44	1.480 (1.007)	<.001	44	1.485 (1.211)	.0025	.9848
(γ -Radiation-baseline)/baseline	44	0.730 (0.821)	—	44	0.224 (0.356)	—	.0003

* *P* calculated for assessing the difference of telomerase activity between baseline and after γ -radiation treatment in cases or controls.
[†] *P* calculated for assessing the difference of telomerase activity between cases and controls.

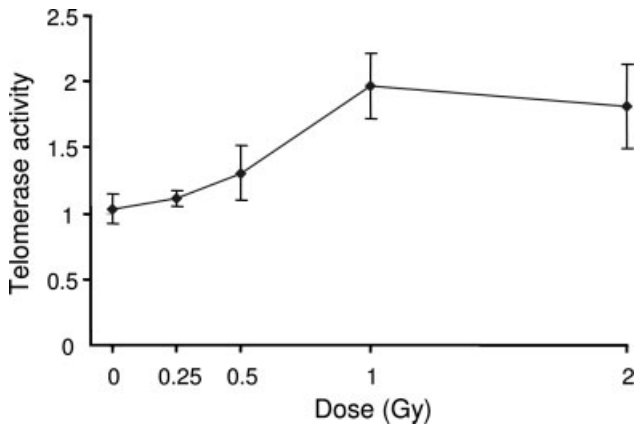


FIGURE 1. Dose-dependent up-regulation of telomerase activity in cultured peripheral blood lymphocytes after γ -radiation. Relative telomerase activity was measured by polymerase chain reaction enzyme-linked immunosorbent assay and normalized to the internal standard sample as described in Materials and Methods. Means and standard deviations were obtained from 3 independent experiments.

between the relative γ -radiation-inducible telomerase activity and lung cancer risk (Table 4). Compared with individuals with the lowest tertile of the ratio, individuals with the second and the highest tertiles of the ratios exhibited significantly elevated risks of lung cancer with adjusted ORs of 12.58 (95% CI: 1.08, 146.86) and 31.08 (95% CI: 2.71, 356.81), respectively (*P* for trend <.001). Due to small sample size, the confidence intervals in the logistic regression analyses (Tables 3, 4) were quite wide. Larger studies are needed to confirm these results and tighten the confidence intervals.

DISCUSSION

Our results demonstrate that 1) the baseline telomerase activity in cultured PBLs was higher in controls than in cases; 2) cultured PBLs from cases exhibited significantly higher γ -radiation inducibility of telomerase activity than those from controls; and 3) higher ratio of net increased to baseline telomerase activity in PBLs was associated with a significantly increased risk of lung cancer.

TABLE 3
Relative Risk Estimates of Lung Cancer for the Ratio of Telomerase Activity Dichotomized by the 75th Percentile Value in Controls

Ratio of telomerase activity γ -radiation-baseline/baseline	Cases no. (%)	Controls no. (%)	OR (95% CI)*
Low	21 (47.73)	34 (77.27)	1 (Reference)
High	23 (52.27)	10 (22.73)	4.71 (1.37, 16.21)

OR indicates odds ratio; CI, confidence interval.
* Adjusted by age, gender, and smoking status.

TABLE 4
Relative Risk Estimates of Lung Cancer for the Ratio of Telomerase Activity by Tertiles in Controls

Ratio of telomerase activity γ -radiation-baseline/baseline	Cases no. (%)	Controls no. (%)	OR (95% CI)*
1st Tertile	1 (2.27)	15 (34.09)	1 (Reference)
2nd Tertile	15 (34.09)	15 (34.09)	12.58 (1.08, 146.86)
3rd Tertile	28 (63.64)	14 (31.82)	31.08 (2.71, 356.81)
<i>P</i> for trend			<i>P</i> <.001

OR indicates odds ratio; CI, confidence interval.
* Adjusted by age, gender and smoking status.

merase activity than those from controls; and 3) higher ratio of net increased to baseline telomerase activity in PBLs was associated with a significantly increased risk of lung cancer.

We used a commercial telomerase PCR ELISA kit that has been utilized by numerous studies for measuring telomerase activity in all types of samples. We tested the variability of this assay in our laboratory during the process of establishing the dose-response curve (Fig. 1). The coefficient of variation (CV) was 12% for the assay from triplicates and 3 independent experiments, which was much lower than interindividual differences in telomerase activity. In the case-control study, the assay was performed in a single 96-well plate and the cases and controls were ran-

domly distributed on the plate. Therefore, there was no interassay variation in this setting. In addition, the measuring variation should randomly and equally affect cases and controls. Furthermore, the scale of the risk estimates, the significant dose-response relation between the 2nd and 3rd tertile of telomerase activity and cancer risk would indicate that the observed associations are not due to measurement bias.

It has been well documented that quiescent PBLs from healthy individuals have very low levels of telomerase activity.^{19–21} However, a number of studies have shown that it was possible to detect telomerase-positive circulating epithelial cancer cells in patients with various solid tumors^{22–28} and the detectability of telomerase activity in peripheral blood correlated with increasing tumor stage. Therefore, the detection of telomerase activity in PBLs has been proposed as a diagnostic or prognostic marker for solid cancers. However, there have not been any reports showing that quiescent PBL itself from cancer patients exhibits increased telomerase activity. Instead, a previous study²⁹ showed that telomerase activity of peripheral blood mononuclear cells (PBMCs, including lymphocytes and monocytes) in patients with laryngeal carcinoma was significantly lower than that in healthy controls. Consistent with this study, we also observed that the baseline telomerase activity in cultured PBLs was higher in controls than in cases, although the difference did not reach statistical significance in our study. However, we had similar findings in another independent case-control study in which the baseline telomerase activity was $\approx 20\%$ higher in healthy controls than in bladder cancer cases (data not shown). A common experimental procedure for these studies is that both used cultured peripheral blood cells in the presence of phytohemagglutinin (PHA) rather than nonstimulated quiescent cells. Previous studies have consistently shown that telomerase activity was dramatically induced when PBMCs or PBLs were cultured in the presence of PHA.^{20,30,31} The telomerase activity increased gradually from 24 to 72 hours and then remained stable for 96 hours.³⁰ In our study the baseline telomerase activity was measured at 84 hours after culturing with PHA, when the telomerase activity should reflect the stable activity in PBL for each individual. It is intriguing why the baseline telomerase activity was lower in PBLs from cancer patients than in those from healthy controls. Ge et al.²⁹ showed that although PHA stimulated both proliferation and telomerase activity of PBMC, the telomerase activity was not strictly proportional to the proliferative response to PHA. Kosciolk and Rowley³² used a classic twin study design to examine the role of genetic factors on telomerase activity in PHA-stimulated PBLs. The

heritability calculated was 0.814, suggesting that PHA-inducible telomerase activity is determined primarily by genetic rather than by environmental factors. It is likely that a group of genes determine the baseline level of telomerase activity and its inducibility, which help to maintain telomere structure and genetic stability. We previously demonstrated that telomere in PBLs were significantly shorter in patients with head and neck, lung, kidney, and bladder cancers than in healthy controls.¹⁸ The lower baseline telomerase activity in cases than in controls (a difference of 0.266 in absolute value and a 21.8% reduction relatively) suggest that cases have a diminished capability to compensate for the shortened telomerase length, and both events synergistically lead to increased genetic instability and hence increased cancer risk. The more interesting finding of this study was that cultured PBLs from cases exhibited higher inducibility of telomerase activity than those from controls, which translated to a 4.71-fold increased lung cancer risk by the higher ratio of net γ -radiation-induced telomerase activity to baseline telomerase activity. γ -Radiation is a potent mutagen that induces DNA damage, cell cycle arrest and apoptosis in a wide variety of cells. Numerous studies have used γ -radiation as a challenging mutagen and demonstrated that deficiency in γ -radiation-induced DNA damage, cell cycle control, and apoptosis pathways were potential risk factors for a number of cancers, including lung cancer.^{33–38} For example, we previously showed that the γ -radiation-induced DNA damage was significantly higher in lung cancer cases and was associated with a 2.32-fold elevated risk of lung cancer. In addition, the median γ -radiation-induced cell cycle delay in S phase and G2 phase was significantly shorter in cases than in controls, and the shorter S and G2 delay resulted in 4.54- and 1.85-fold increased risks, respectively, of lung cancer.³⁹ This current study is the first epidemiologic study to suggest that γ -radiation-induced telomerase activity may also be a susceptibility factor for lung cancer. Telomerase activation is a prerequisite for cell immortalization and tumorigenesis. It is plausible that a highly inducible telomerase may act in concert with carcinogen exposure to provide a growth advantage to cells that have increased DNA damage, deficient cell cycle checkpoints, or reduced apoptotic response, resulting in elevated cancer risk.

The biological mechanism for the higher γ -radiation-inducibility of telomerase in cases than in controls is not clear. We speculate that it may be related to a γ -radiation-induced p53 damage response pathway. We previously measured γ -radiation-induced p53 protein levels in lymphoblastoid cell lines from

30 lung cancer patients and 22 healthy controls obtained from the same case-control population as those used in the present study.³³ The mean p53 protein expression at baseline was higher in cases than controls ($P = .011$). After γ -radiation treatment, however, the ratio of p53 expression in treated to untreated cells was significantly higher in controls than in cases ($P = .03$). Several studies have shown that p53 expression down-regulated telomerase activity.⁴⁰⁻⁴³ There is an inverse relation between p53 level and telomerase activity before and after γ -radiation from these 2 studies. At baseline, p53 expression was higher in cases than the controls, leading to lower baseline telomerase activity in cases due to higher p53 repression in cases. After γ -radiation, higher induction of p53 expression in controls would result in higher inducibility of telomerase activity in cases due to less inhibition by p53 in cases.

The major limitation of this study is the problem of 'reverse causation,' ie, whether the variations in baseline and γ -radiation-induced telomerase activity are the results of disease or prior environmental exposure. We attempted to address this limitation by evaluating the impact of smoking status, smoking intensity (pack-years), and tumor stage on telomerase activity and did not find significant associations (data not shown). In addition, we also collected information in our controls and cases with regard to chronic obstructive pulmonary disease (COPD) and emphysema and we did not find an association between telomerase activity and these chronic lung diseases. Due to small sample size, these exploratory stratified analyses were not conclusive and larger studies are warranted to confirm our observations. Furthermore, because 'reverse causation' is an inherited limitation of retrospective case-control studies, prospective studies are desired to address this question. Nevertheless, Kosciolk and Rowley³² compared the PHA-induced telomerase activity in PBLs in monozygotic and dizygotic twins and calculated a heritability of 0.814, providing strong evidence that telomerase activity in PBLs is highly heritable and therefore supporting the use of telomerase activity in PBLs as a cancer susceptibility factor.

In conclusion, this pilot study showed that the telomerase activity in PHA-stimulated PBLs was lower in lung cancer cases than in healthy controls. This is the first study to demonstrate that the γ -radiation-inducible telomerase activity is associated with an increased risk of lung cancer. The ratio of net γ -radiation-induced telomerase activity to baseline telomerase activity in cultured PBLs may be a novel phenotypic biomarker for determining susceptibility to lung cancer.

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